

## Short communication

Prevalence of *Toxoplasma gondii* in dogs from Sri Lanka  
and genetic characterization of the parasite isolatesJ.P. Dubey<sup>a,\*</sup>, R.P.V.J. Rajapakse<sup>b</sup>, R.R.M.K.K. Wijesundera<sup>b</sup>, N. Sundar<sup>a</sup>,  
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## Abstract

The prevalence of *Toxoplasma gondii* in 86 street dogs from Sri Lanka was determined. Antibodies to *T. gondii* were assayed by the modified agglutination test (MAT) and found in 58 (67.4%) of 86 dogs with titers of 1:20 in eight, 1:40 in four, 1:80 in 10, 1:160 in 22, 1:320 in six, 1:640 in five, and 1:1280 or higher in three. Hearts, tongues, and brains (either separately or pooled) of 50 dogs with MAT titers of 1:40 were selected for isolation of *T. gondii* by bioassays in mice. For bioassays, canine tissues were digested in pepsin and homogenates were inoculated subcutaneously into mice; the mice receiving canine tissues were examined for *T. gondii* infection. In all, *T. gondii* was isolated from 23 dogs. Interestingly, dog organs varied in their capacity to induce *T. gondii* infection in mice, muscles producing more positive results than the brain. The *T. gondii* isolates obtained from 23 seropositive dogs were PCR-RFLP genotyped using polymorphisms at 10 nuclear markers including SAG1, SAG2, SAG3, BTUB, GRA6, c22-8, c29-2, L358, PK1, a new SAG2, and an apicoplast marker Apico. Mixed infection with two genotypes was observed in one dog. Four genotypes were revealed, including three unique genotypes in addition to one belonging to the predominant Type III lineage. The 24 isolates were designated as TgDgSl 1–24.

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Keywords: *Toxoplasma gondii*; Dogs; Sri Lanka; Bioassays; Antibodies; Genotype

## 1. Introduction

*Toxoplasma gondii* infections are widely prevalent in human beings and animals worldwide (Dubey and Beattie, 1988). Humans become infected post-natally by ingesting tissue cysts from undercooked meat, consuming food or drink contaminated with oocysts, or by accidentally ingesting oocysts from the environment

(Dubey and Beattie, 1988). However, only a small percentage of exposed adult humans develop clinical symptoms (Dubey and Beattie, 1988). It is unknown whether the severity of toxoplasmosis in immunocompetent persons is due to the parasite strain, host variability, or to other factors.

*T. gondii* isolates have been classified into three genetic Types (I, II, III) based on restriction fragment length polymorphism (RFLP) (Howe and Sibley, 1995; Howe et al., 1997) and until recently, *T. gondii* was considered to be clonal with very little genetic variability. Based on recent studies, a higher genetic variability has been revealed than previously reported

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(Ajzenberg et al., 2004; Ferreira et al., 2006; Khan et al., 2006; Lehmann et al., 2006).

We have initiated a study on the genetic diversity of *T. gondii* worldwide with ultimate objective to understanding the protein variability among strains for immunoprophylaxis. In the present study, we attempted to isolate and characterize *T. gondii* from dogs from Sri Lanka. We also examined distribution of *T. gondii* in tissues of asymptomatic dogs to improve biological diagnosis.

## 2. Materials and methods

### 2.1. Naturally infected dogs

Eighty-six street dogs that were caught by the municipality were euthanized by intravenous injection of sodium thiopentone. The study group comprised of 48 males and 38 females and were of mixed breed and different age groups. At necropsy, brain, heart, tongue and blood samples were collected and kept at 4 °C until sent refrigerated by air to Beltsville, MD. The samples were received in March (batch 1) and June (batch 2) 2006. Six days elapsed between the killing of dogs and the receipt of samples in Beltsville.

### 2.2. Serological examination

Sera of dogs were tested for *T. gondii* antibodies using two-fold serum dilutions from 1:20 to 1:1280 with the modified agglutination test (MAT) as described by Dubey and Desmonts (1987).

### 2.3. Bioassay of canine tissues for *T. gondii* infection

Tissues of 50 dogs (24 in batch 1 and 26 in batch 2) with titers of 1:40 or higher were bioassayed for *T. gondii* infection in mice, 1–3 days after the results of serologic examination were available (Table 1). For the dogs in batch 1, brains were bioassayed separately and the hearts and tongues from each were pooled together.

For batch 2 dogs, brains, hearts, and tongues were bioassayed separately for each dog into out-bred female Swiss Webster mice obtained from Taconic Farms, Germantown, New York, as described by Dubey et al. (2002). Each tissue (20–25 g) was homogenized individually, digested in acidic pepsin, neutralized, and washed (Dubey, 1998); the sedimented homogenate was suspended in antibiotic saline and an aliquot was inoculated subcutaneously into four mice (Table 1).

The mice receiving canine tissues were examined for *T. gondii* infection. Mice were bled on days 40–42 post-inoculation (p.i.) and a 1:25 dilution of serum from each mouse was tested for *T. gondii* antibodies with the MAT. Mice were killed 6-week p.i. and brains of all mice were examined for tissue cysts as described (Dubey and Beattie, 1988). If tissue cysts were not found in seropositive mice, their brain homogenates were inoculated into interferon gamma gene knock out (KO) as described (Dubey and Lindsay, 1998); these KO mice are highly susceptible to intracellular protozoan infections because they lack the cytokine important for developing protective immunity. The inoculated mice were considered infected with *T. gondii* when tachyzoites or tissue cysts were found in tissues.

### 2.4. Genetic characterization

*T. gondii* DNA was extracted from tissues of infected mice from each group and strain typing was initially performed using PCR-RFLP genetic markers SAG1, SAG2, SAG3, BTUB and GRA6 (Dubey et al., 2006). In brief, the target DNA sequences were amplified by multiplex PCR using external primers for all five markers. Multiplex PCR amplified products were then used for nested PCR with internal primers for each marker separately. Nested PCR products were treated with restriction enzymes and resolved in agarose gel by electrophoresis to reveal the RFLP patterns of the isolates. These five markers allow us to quickly characterize all samples and to identify potential mixed infection in dogs. The samples with low DNA concentration and cannot be reliably genotyped by

Table 1  
Prevalence of *T. gondii* infection in dogs from Sri Lanka

Batch number	Number of dogs	Number of seropositive (MAT > 40)	Total bioassay-positive dogs	<i>T. gondii</i> isolation from canine		Genotypes			
				Brain	Muscle	#1	#2	#3	#4
1	36	24	13 <sup>a</sup>	5	12	3	6	5	0
2	50	26	10	3	10	0	5	4	1

<sup>a</sup> Two genotypes were obtained from one dog.

these markers were removed from further analysis. One or two representative DNA extracts from mice infected with the same dog sample were genotyped with six additional genetic markers including c22-8, c29-2, L358, PK1, a new SAG2 and Apico to further identify isolates with high resolution (Dubey et al., 2007a; Su et al., 2006) by the same method described above. Allele types for all isolates were determined based on the RFLP patterns of six reference strains including RH88, PTG, CTG, COUGAR, MAS and TgCatBr5 (Su et al., 2006). These reference strains allow us to capture all known alleles for each marker and to identify potential unique alleles in new samples.

### 3. Results

#### 3.1. Seroprevalence

In total, antibodies to *T. gondii* were found in 58 (67.4%) of 86 dogs. Antibody titers of dogs in batch 1 were 1:40 in two, 1:80 in five, 1:160 in six, 1:320 in four, 1:640 in four, and 1:1280 or higher in three dogs. Antibody titers of dogs in batch 2 were 1:20 in eight, 1:40 in two, 1:80 in five, 1:160 in 16, 1:320 in two, and 1:640 in one.

#### 3.2. Isolation of *T. gondii* from canine tissues

*T. gondii* was isolated from 23 dogs by bioassay in mice (Table 1). The isolation rate was higher from dogs from batch 1 (54.1%) than dogs from batch 2 (38.4%).

From the batch 1 dogs, *T. gondii* was isolated from 13 dogs; from the brains and muscles of three, brain alone of one, and muscles of nine (Table 2). From batch 2 dogs, *T. gondii* was isolated from 10 dogs; from brain, heart, and tongue of one, from tongue alone of two, and heart alone of seven (Table 3). Overall, *T. gondii* was isolated from brains of eight dogs and muscles of 22 dogs.

None of the SW mice inoculated with canine tissues became ill or died due to toxoplasmosis and very few tissue cysts were found in their brains, indicating low pathogenicity of the isolates for mice. However, KO mice inoculated with brains of seropositive mice died of toxoplasmosis and tachyzoites were found in their lungs.

#### 3.3. *T. gondii* genotypes and strain designation

The *T. gondii* isolates were designated as TgDgSI 1–24 (Tables 2 and 3); two genetically different isolates were obtained from one dog (no. 19).

The 24 *T. gondii* isolates from dogs were PCR-RFLP genotyped using polymorphisms at 10 nuclear markers including SAG1, SAG2, SAG3, BTUB, GRA6, c22-8, c29-2, L358, PK1, a new SAG2, and an apicoplast marker Apico (Tables 2 and 3). Four genotypes were identified and summarized in Tables 1 and 4. Of the four genotypes identified, there were three isolates with genotype #1 profile (TgDgSI 5, 6 and 10), typical of the Type III lineage. Eleven isolates (TgDgSI 1, 2, 3, 7, 8, 9, 15, 17, 19, 20 and 24) were of the genotype #2 profile and had the combination of alleles I, II and III alleles from different loci. Nine isolates (TgDgSI 4, 11, 12, 13,

Table 2  
Isolation of *T. gondii* from dogs of batch no. 1

Experiment no. TX 246 and dog no.	MAT titer	Isolation in mice <sup>a</sup> from canine		<i>T. gondii</i> strain designation	Genotypes	
		Brain	Heart + tongue		SAG1, SAG2, SAG3, BTUB, GRA6	c22-8, c29-2, L358, PK1, new SAG2, Apico
4	80	1	0	TgDgSI 1	II or III, II, II, II, II	II, II, I, II, II, I
6	80	0	2	TgDgSI 2	II or III, II, II, II, II	II, II, I, II, II, I
13	640	0	3	TgDgSI 3	II or III, II, II, II, II	II, II, I, II, II, I
16	640	0	1	TgDgSI 4	u-1, II, III, III, II	II, III, II, u-2, II, I
17	640	0	3	TgDgSI 5	II or III, III, III, III	III, III, III, III, III, III
19	320	4	–	TgDgSI 6	II or III, III, III, III	III, III, III, III, III, III
19	320	–	2	TgDgSI 7	II or III, II, II, II, II	II, II, I, II, II, I
22	160	4	4	TgDgSI 8	II or III, II, II, II, II	II, II, I, II, II, I
24	320	2	4	TgDgSI 9	II or III, II, II, II, II	II, II, I, II, II, I
25	80	2	2	TgDgSI 10	II or III, III, III, III, III	III, III, III, III, III, III
30	1280	0	2	TgDgSI 11	u-1, II, III, III, II	II, III, II, u-2, II, I
31	160	0	4	TgDgSI 12	u-1, II, III, III, II	II, III, II, u-2, II, I
32	320	0	1	TgDgSI 13	u-1, II, III, III, II	II, III, II, u-2, II, I
35	1280	0	2	TgDgSI 14	u-1, II, III, III, II	II, III, II, u-2, II, I

<sup>a</sup> Number of mice infected with *T. gondii* of four mice inoculated with canine tissues.

Table 3

Isolation of *T. gondii* from dogs of batch no. 2

Experiment no. TX 256 and dog no.	MAT titer	Isolation in mice <sup>a</sup> from canine			<i>T. gondii</i> strain designation	Genotypes	
		Brain	Heart	Tongue		SAG1, SAG2, SAG3, BTUB, GRA6	c22-8, c29-2, L358, PK1, new SAG2, Apico
2	160	0	0	2	TgDgSI 15	II or III, II, II, II, II	II, II, I, II, II, I
11	160	0	2	0	TgDgSI 16	u-1, II, III, III, II	II, III, II, u-2, II, I
15	160	1	0	3	TgDgSI 17	II or III, II, II, II, II	II, II, I, II, II, I
16	160	1	4	4	TgDgSI 18	u-1, II, III, III, II	II, III, II, u-2, II, I
22	160	0	2	0	TgDgSI 19	II or III, II, II, II, II	II, II, I, II, II, I
23	40	0	4	0	TgDgSI 20	II or III, II, II, II, II	II, II, I, II, II, I
25	80	0	1	0	TgDgSI 21	u-1, II, III, III, II	II, III, II, II, II, I
33	160	0	1	0	TgDgSI 22	u-1, II, III, III, II	II, III, II, u-2, II, I
51	160	0	2	4	TgDgSI 23	u-1, II, III, III, II	II, III, II, u-2, II, I
52	80	2	4	4	TgDgSI 24	II or III, II, II, II, II	II, II, I, II, II, I

<sup>a</sup> Number of mice infected with *T. gondii* of four mice inoculated with canine tissues.

14, 16, 18, 22 and 23) were of genotype #3 profile and had the combination of different alleles with an unique allele (u-1) at SAG1 locus and an unique allele (u-2) at PK1 locus. One isolate (TgDgSI 21) was designated genotype #4 as it differed from genotype #3 only at the locus PK1 with the Type II instead of the u-2 allele. This result was confirmed by re-test of the isolate in an independent experiment. Mixed infection was found in one dog and the isolates were designated as TgDgSI 6 (genotype #1) and TgDgSI 7 (genotype #2).

#### 4. Discussion

##### 4.1. Tissue localization

In the present study *T. gondii* was isolated from tissues of 23 of 50 dogs with MAT titers of 1:40 or

higher. No definitive statement can be made regarding the valid cut-off MAT titer for *T. gondii* infection in dogs. Little is known of the tissue distribution of *T. gondii* in dogs because we did not bioassay dogs with MAT titers of lower than 1:40. *T. gondii* is considered to have an affinity for encystment in neural tissue but this assumption is based on infections in mice. In the present study, *T. gondii* was isolated more frequently from muscles than from the brains of dogs using an equal amount of tissue for bioassay in mice. These data indicates that muscle tissue should be included for bioassay of canine tissues for *T. gondii* infection. The isolation of *T. gondii* from tongue tissue of five of the 10 bioassay-positive dogs suggests that tongue can be used to isolate *T. gondii* from dead dogs in case facilities are not available to obtain heart and brain.

Table 4

Summary of genotyping of *T. gondii* from dogs from Sri Lanka

Genotype	Genetic markers											Isolate ID
	SAG1	SAG2	SAG3	BTUB	GRA6	c22-8	c29-2	L358	PK1	New SAG2	Apico	
Reference	I	I	I	I	I	I	I	I	I	I	I	RH88
Reference	II or III <sup>a</sup>	II	II	II	II	II	II	II	II	II	II	PTG
Reference	II or III	III	III	III	III	III	III	III	III	III	III	CTG
Reference	I	II	III	II	II	II	u-1	I	u-2	II	I	COUGAR
Reference	u-1	I	III	III	III	u-1	I	I	III	II	I	MAS
Reference	I	III	III	III	III	I	I	I	u-1	III	I	TgCatBr5
#1	II or III	III	III	III	III	III	III	III	III	III	III	TgDgSI 5, 6, 10
#2	II or III	II	II	II	II	II	II	I	II	II	I	TgDgSI 1, 2, 3, 7, 8, 9, 15, 17, 19, 20, 24
#3	u-1	II	III	III	II	II	III	II	u-2	II	I	TgDgSI 4, 11, 12, 13, 14, 16, 18, 22, 23
#4	u-1	II	III	III	II	II	III	II	II	II	I	TgDgSI 21

<sup>a</sup> At SAG1 locus, Types II and III are indistinguishable.

#### 4.2. Mouse pathogenicity of canine isolates of *T. gondii*

*T. gondii* isolates differ markedly in their virulence to out bred mice. Isolates of *T. gondii* were considered virulent when mice inoculated with tachyzoites or bradyzoites died within 28 days p.i. Virulence to mice depends on several factors including the stage of the parasite, route, dose, types of mice used, host, and the strain of the parasite. In general, *T. gondii* isolates from North America are less pathogenic to mice than isolates from South America, in particular Brazil (Dubey et al., 2002, 2007a). However, little is known of the mouse pathogenicity of *T. gondii* isolates from Asia. Results of our previous studies indicated that mouse virulent strains are present in apparently clinically normal dogs from Brazil (Dubey et al., 2007b), Colombia, South America (Dubey et al., 2007c). Results of the present study indicated that *T. gondii* isolates from dogs from Sri Lanka were not pathogenic for mice.

#### 4.3. Genotyping

Genotyping of the 24 *T. gondii* isolates revealed four genotypes (Tables 2 and 3). Genotype #1 is indistinguishable from the Type III lineage that has a worldwide distribution. However, 20 of the 24 isolates belong to two other groups (genotypes #2 and #3), suggesting that these two genotypes may be predominant in Sri Lanka. Isolate TgDgSl 21 represents the fourth genotype and it differs from genotype #3 only at the locus PK1, indicating they are closely related. Interestingly, mixed infection was also observed from one of the 23 seropositive dogs. Comparing with previously published genotyping data from isolates of dogs (Dubey et al., 2007b,c), cats (Dubey et al., 2007d; Su et al., 2006) and chickens (Dubey et al., 2007a) showed that genotypes #2 and #3 from Sri Lanka are unique. However, genotype #4 was found in dogs from Colombia (Dubey et al., 2007c), cats from China (Dubey et al., 2007d), and chickens from Pará, Brazil (Dubey et al., 2007a), indicating the widespread of genotype #4. A recent population genetic study based on microsatellite markers showed that there were two genotypes, namely WW and RW in chicken isolates from Sri Lanka (Lehmann et al., 2006). However, here in this study, PCR-RFLP markers are used instead and the results cannot be compared directly. Therefore, it is not clear how genotypes WW and RW relate to genotypes #2, #3 and #4 among isolates from Sri Lanka. In summary, the genotyping result for dogs in Sri Lanka indicates that

the diversity of *T. gondii* may be at low to intermediate level in the region.

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